*U.S. Department of Health and Human Services*
*National Institutes of Health*
*National Institute of Dental and Craniofacial Research*
*Division of Intramural Research*

# ANNUAL REPORT SUMMARY

## 2003

# Department of Health and Human Services
# National Institutes of Health

# National Institute of Dental and Craniofacial Research

## Division of Intramural Research

# Annual Report Summary

## 2003

For Administrative Use Only

Fiscal Year 2003 Annual Report

National Institute of Dental and Craniofacial Research
Division of Intramural Research

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# Overview

Henning Birkedal-Hansen, Scientific Director

# National Institute of Dental and Craniofacial Research
# Division of Intramural Research
## Overview

The Division of Intramural Research (DIR) enjoyed a productive and prosperous year in FY2003. This report summarizes the outstanding scientific achievements of our scientists over the past year and highlights our scientific findings. Our research resulted in approximately 300 publications, many in leading international journals. This document is organized to provide a summary of the research conducted by our six Branches and four single standing units.

The Oral Infection and Immunity Branch was reviewed by the Board of Scientific Counselors in June of 2003. The Branch and its investigators received high marks for their creativity and scientific excellence.

The Division recruited a new Clinical Director, Dr. Thomas Hart, a periodontist and geneticist, who will join the program in October 2003. Dr. Hart brings many strengths to this position, an excellent and internationally regarded research program and a broad basis in both dental and medical research, and a great deal of enthusiasm. We are excited over Dr. Hart assuming leadership of our clinical research infrastructure.

The Division conducted three tenure track searches which resulted in the appointment of three outstanding scientists, Songtao Shi, DDS, a pediatric dentist in the Craniofacial and Skeletal Diseases Branch, Wenyuan Chen, MD in the Oral Infection and Diseases Branch and Matthew Hoffman, DDS in the Craniofacial Developmental and Regeneration Branch.

Renovation and infrastructure improvements proceeded with full force in the basement of Building 30 for creation of a new state-of-the-art animal facility which will open in October 2003. In addition, the renovation of the H. Trendley Dean Conference Room was completed and an extra floor was added on top of the conference room which will provide space for additional activities. The first two phases of renovation on the fourth floor are in progress.

There are currently 28 Senior Investigators and 6 Tenure Track Investigators in the DIR. The Division is focusing on rebuilding in several areas by recruitment of tenure track positions in pulpal stem cell biology, structural biology, host-microbial interactions and mucosal immunology.

DIR scientists also were highly active in organizing scientific meetings and workshops, being invited to present at international scientific meetings and giving invited presentations at universities across the country. In addition, our scientists continue to develop an extensive network of national and international collaborative projects with colleagues in the US and abroad. These activities are detailed annually in a separate document entitled "Interactions with the Scientific Community."

Henning Birkedal-Hansen
Scientific Director

# Craniofacial Developmental Biology and Regeneration Branch

Kenneth Yamada
Hynda Kleinman
Yoshihiko Yamada

# CRANIOFACIAL DEVELOPMENTAL BIOLOGY AND REGENERATION BRANCH
## 2003

The Craniofacial Developmental Biology and Regeneration Branch (CDBRB) focuses on four general objectives: understanding the mechanisms of normal and defective craniofacial development at genetic, molecular, and cellular levels; characterizing related processes in tissue repair and cancer; discovering novel genes, biologicals, and biomimetics relevant to diagnosis, repair, and therapy; and developing biologically based methods to replace or regenerate tissues that are defective or damaged. We place particular emphasis on research characterizing the interactions between cells and extracellular molecules. Project areas include identification of mechanisms of morphogenesis, characterization of the gene regulation, organization, and function of extracellular matrix molecules and their receptors, and analysis of signaling pathways from the cell surface to the nucleus. These ongoing basic research innovations should ultimately provide the basis for novel translational and patient-oriented applications. Our researchers continue to generate exciting research advances and to receive international recognition. We also continue to place high priority on training younger scientists to become independent leaders in academia and industry. In addition, we provide extensive service and citizenship activities on behalf of NIDCR, NIH, and our research fields.

A major new development in our Branch will be the establishment of a tenure-track position in developmental biology of the mammalian salivary gland to take advantage of new research opportunities in this field. Matthew P. Hoffman will be appointed to this position. Dr. Hoffman is a dentist-scientist with extensive research experience in the study of branching morphogenesis and growth factor receptors during salivary development. He will move out of the Cell Biology Section into this independently funded position.

Over the past fiscal year, CDBRB researchers have made substantial progress and exciting scientific breakthroughs, as reflected in the 59 publications in our annual report bibliography. Several selected research advances and a clinical trial are highlighted below. More comprehensive summaries of the major new findings in our Branch can be found in the Project reports of each Section.

All three CDBRB Sections initiated genome projects to catalogue genes expressed in oral and craniofacial tissues and to discover novel genes important for tooth, oral, and craniofacial development. In one project, CDBRB researchers discovered and characterized the gene for ameloblastin, a developmentally regulated, tooth-specific extracellular matrix protein. They also analyzed mouse tooth germ and craniofacial patterns of gene expression using cDNA microarrays. Several new tissue-specific genes have been identified, including a novel Krüppel-like zinc-finger factor that was named epiprofin, which is predominantly expressed by epithelium of developing tooth, hair follicle, and limb bud, and which functions to promote cell proliferation. The roles of epiprofin in the development of these tissues are being characterized in mice.

In studies of salivary gland development, gene expression profiles of multiple embryonic stages using microarray and laser micro-dissection/SAGE approaches by the Cell Biology and Developmental Mechanisms Sections have identified a variety of additional genes that may be important in salivary development. CDBRB members also served as Project Officers of a major contract with Washington University, St. Louis, in association with researchers at Johns Hopkins University and Necker-Enfant Malades Hospital, to discover human craniofacial genes that are active in specific tissues during early development. All clones, cDNA libraries, and antibodies will continue to be made freely available to dental, craniofacial, and other investigators to promote research in the area.

Our knowledge about gene regulation and the extracellular matrix is being used to examine pathology in animal models and in human diseases. For example, the Molecular Biology Section has created gene knockout mice for perlecan and identified mutations in the perlecan gene in two human diseases: severe neonatal lethal dyssegmental dysplasia (Silverman-Handmaker type) and the relatively mild Schwartz-Jampel syndrome with chondrodystrophic myotonia. They found that perlecan is essential for cartilage development and plays an important role in neuromuscular junction function. They have created a mouse model that rescues the lethal phenotype of perlecan-null mice by expressing recombinant perlecan in cartilage to study the mechanism of the myotonia and to develop potential reagents for therapy.

Laminin and laminin peptides have been implicated by Molecular and Cell Biology Section members in differentiation, tumor growth, and metastasis. The role of laminin in development is being explored in animal models by creating gene-targeted mice. Active sites of laminin chains have been identified using synthetic peptides, and their cellular receptors have been characterized. Many peptides have potent cell type-specific effects on adhesion, angiogenesis, salivary gland differentiation, and metastasis. A mimetic of one of the active peptides reduces tumor growth and angiogenesis in vivo. This and other peptides may have therapeutic uses.

The Cell Biology Section has identified potent extracellular regulators of cell migration and tumor metastasis. Ongoing studies on thymosin beta-4 have established a role in cell migration. It decreases inflammation and accelerates wound repair in the cornea and in dermal wounds in aged and diabetic animals, suggesting its potential for promoting human wound healing. A phase I clinical trial has now been completed with thymosin beta-4. Furthermore, this molecule induces keratinocyte expression of laminin-5, which is reduced in patients with epidermolysis bullosa. A phase IIA clinical trial will include these patients. Unexpectedly, thymosin beta-4 promoted hair growth in rodents. It acts by increasing stem cell migration, differentiation, and protease production. It also promotes tumor metastasis and up-regulates VEGF by binding to the VEGF promoter. Since it enters the cell nucleus, it also acts as a transcription factor.

Successful regeneration of salivary gland function is likely to require re-establishing the complex branched organization of acini to provide enough epithelial surface area to meet saliva fluid requirements. The Cell Biology and Developmental Mechanisms Sections have consequently focused on determining the mechanisms and genes regulating early salivary gland branching morphogenesis. FGF receptors, FGFs, and proteases regulate the number and size of buds formed in the early gland. The PI 3-kinase pathway was also found to be important in early salivary gland development. The Developmental Mechanisms Section recently discovered that local, developmentally regulated expression of fibronectin was required for effective salivary, lung, and kidney branching morphogenesis. The mechanism involves local suppression of cadherin localization by fibronectin fibrils and a conversion from cell-cell to cell-matrix adhesions in developing clefts. Other studies characterized cell adhesions to matrix-derivatized biomaterials. These approaches are building the knowledge base necessary to develop creative therapeutic approaches for the repair or replacement of salivary glands and other tissues. For example, CDBRB members are collaborating with the Gene Therapy and Therapeutics Branch to develop a first-generation artificial salivary gland.

Integrins are major mediators of cell interactions with the extracellular matrix. The Developmental Mechanisms Section has dissected mechanisms of integrin-mediated function by identifying key components of integrin adhesive complexes and exploring how signal transduction is initiated. Vinculin accumulation was found to be an important step in generating force for cell migration in early cell-matrix adhesions. A well-characterized molecule involved in initiating integrin signaling is the focal adhesion kinase (FAK). Experimentally inducing a dimer of FAK was sufficient to initiate signaling, but efficient MAP kinase activation required the formation of larger complexes. A later signaling step downstream of FAK found to be important for migration was tyrosine phosphorylation of the CrkII adaptor protein located in integrin complexes.

Besides publishing extensively in leading journals, our Branch distributes its research materials widely by licensing, donating to repositories, and providing numerous gifts to research colleagues. Products generated by CDBRB members that were licensed by companies included Matrigel, invasion substrates, and monoclonal antibodies against integrins. CDBRB has donated hundreds of cDNA clones to the ATCC and completed more than 70 formal Material Transfer Agreements with extramural researchers over the past year to provide our reagents as gifts. Members of the Branch have also received support from outside organizations. NASA provided funds to study salivary gland cell differentiation in microgravity, and external salary support for postdoctoral members of CDBRB has come from several different sources.

CDBRB members continue to be invited as featured speakers at a variety of international meetings and symposia. Examples from this past fiscal year included Hynda K. Kleinman as a speaker at the Gordon Conference on Repair and Regeneration, Kenneth M. Yamada as a keynote speaker at the 2nd International Symposium on Extracellular Matrix in Brazil, and Yoshihiko Yamada as a speaker at the Cartilage Gordon Conference. Our members continue to serve on the editorial boards of a number of leading journals. Examples include J. Cell Biology (K. Yamada, Editor, and H. Kleinman, board member); Cancer Research (H. Kleinman, Associate Editor); Matrix Biology (Y. Yamada and K. Yamada, Associate Editors); J. National Cancer Institute (H. Kleinman, Associate Editor); J. Cellular Physiology and Cell Communication and Adhesion (K. Yamada, Associate Editor); J. Biological Chemistry (Y. Yamada, board member), and a variety of other editorial boards. Members also serve on various review panels, on the Board of the Metastasis Research Society (H. Kleinman), and on the Councils of the International Society for Matrix Biology (K. Yamada) and the American Society for Matrix Biology (H. Kleinman and K. Yamada). CDBRB members provide extensive service on more than two-dozen NIH and NIDCR committees, including the NIH Senior Biomedical Research Service Policy Board (K. Yamada) and the NIH Central Tenure Committee (H. Kleinman).

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# Craniofacial and Skeletal Diseases Branch

Pamela Gehron Robey
Larry W. Fisher
Marian F. Young

# CRANIOFACIAL AND SKELETAL DISEASES BRANCH
# 2003

The Branch has continued to concentrate its efforts on gaining an in-depth understanding of the skeleton, defined as bone, cartilage, teeth, and their associated soft tissues. The rationale for this emphasis is based not only on the critical role that skeletal tissues play in oral health, but also on the increasing incidence of skeletal disorders, and the lack of efficacious therapy for most of these disorders. The goal is to apply cutting edge basic, translational and clinical research in order to impact current medical practice. To accomplish this goal, studies focus on the cells, genes and macromolecules of skeletal tissues in order to elucidate the fundamental mechanisms that govern development, growth and maintenance of the normal skeleton, and to determine the pathogenic mechanisms in genetic and acquired skeletal disorders. These studies are performed by three Principal Investigators and a newly appointed Tenure-Track Investigator who are highly collaborative, not only within the Branch, NIDCR and NIH, but also worldwide. Each group focuses on specific aspects of skeletal metabolism and has positioned the Branch to address major physiological questions, to devise new hypotheses, and to test them through in vitro and in vivo analyses, and through clinical observation.

The Skeletal Biology Section, directed by Dr. Pamela Gehron Robey, has focused on stem cells that form hard tissues, in particular, bone marrow stromal stem cells (BMSSCs). Our previous studies have provided evidence for the existence of adult human bone marrow stromal stem cells (BMSSCs). In a recent study, we determined that forced telomerase expression was able to accelerate calcium accumulation of human BMSSCs under osteogenic inductive conditions. Similarly, xenogeneic transplantation of telomerase-expressing BMSSCs (BMSSC-Ts) yielded ectopic bone formation at 2 weeks post-transplantation, 2–4 weeks earlier than typically seen with BMSSCs transfected with empty vector (BMSSC-Cs). The findings also showed that telomerase can accelerate cell cycle progression from G1-to-S phase by increasing the expression of G1 regulating genes including cyclin D3, cyclin E1, E2F-4, and DP2, associated with hyperphosphorylation of retinoblastoma (pRb), and enhance osteogenic differentiation of BMSSCs, because of the upregulation of CBFA1, osterix, and osteocalcin. While the existence of post-natal stem cells in bone marrow is firmly established, their localization has eluded detection do to the lack of useful definitive markers. We have recently shown that BMSSCs localize to microvasculature in situ, based on their expression of the perivascular markers, STRO-1 and CD146 (MUC-18), using immunofluorescence and magnetic activated cell sorting, similar to what was found for dental pulp stem cells (DPSCs). The finding that different mesenchymal stem cells share a common perivascular niche may have further implications in understanding the factors that regulate the formation of mineralized matrices and other connective tissues.

The Molecular Biology of Bones and Teeth Unit, directed by Dr. Marian F. Young, continues to investigate the function and regulation of matrix proteins deposited in mineralized tissues. Small leucine rich proteoglycans, the SLRPs, are abundant in mineralized tissuses and were the focus of the group. In order to study their function in vivo, animal models deficient in one or more SLRP were created and shown to acquire multiple skeletal defects including early onset osteopenia and osteoarthritis. The goal of theses studies was to investigate the effects of physiological stress on the bones of mutant mice (knockout/KO) unable to make the SLRP called biglycan (bgn). This was done to determine what role bgn plays in regulating bone formation, bone resorption and bone turnover (coupling of the two processes). First, femora from 2 month-old normal and mutant mice were flushed (ablated) to induce rapid and reproducible osteogenesis. Analysis of bone tissue by X-ray and pQCT showed that *bgn* deficient mice had significantly less accumulation of bone compared to normal mice proving that bgn is important for the osteogenesis in vivo. To test whether bgn could be involved in the effects of estrogen depletion on induced bone formation, we analyzed the bones from normal and *bgn* deficient mice after ovariectomy (OVX). *Bgn* deficient mice were resistant to OVX induced trabecular bone loss whereas the normal mice responded as expected and had increased bone turnover. These data support the concept that bgn has dual roles in bone where it modulates both formation and resorption ultimately influencing the bone turnover process. Using bone cells isolated from *bgn* deficient mice we showed that the absence of bgn caused less BMP-4 binding, which reduced the sensitivity of osteoblasts to BMP-4 stimulation. The loss of sensitivity resulted in reduced expression of the osteogenic master gene, *cbfa1*, which, ultimately led to a defect in the differentiation of osteoblasts. Based on these observations we theorize that bgn can modulate BMP-induced bone cell differentiation. This data provides a molecular basis for the osteoporosis noted in the *bgn* KO mice.

The Matrix Biochemistry Unit has continued to define the functions of the SIBLING (Small Integrin-Binding LIgand, N-linked Glycoprotein) family of secreted phosphoproteins. We have observed that the five genes currently known for this family are clustered within a 375,000 basepair region of human chromosome 4 and an even slightly smaller region of mouse chromosome 5. The SIBLINGs have for many years been associated with biomineralization but we have preliminary evidence in epithelial tissues such as salivary glands that they each have at least one function that is not directly related to calcification. With our colleagues in Belguim and Italy, we have extended our previous work in the expression of SIBLINGs in tumors to include bone sialoprotein (BSP) expression in malignant melanoma and dentin matrix protein 1 (DMP1) in lung cancer. This expands the number of cancers for which our current patent application on the levels of SIBLINGs in blood, urine and saliva may be diagnostically useful. Our group has begun to explore the role of these proteins in the activation of specific matrix metalloproteinases (MMPs) in both normal tissues and in cancer.

The research of the newly established Dental Biology Unit, headed by Dr. Songtao Shi is focused on isolation and characterization of orofacial stem cells. We isolated stem cells from human exfoliated deciduous teeth (SHED) and identified SHED as a unique population of post-natal stem cells capable of differentiating into a variety of cell types, including neural cells, adipocytes, as well as odontoblasts. Upon in vivo transplantation, SHED cells were found to be able to induce a significant amount of bone formation, generate dentin, and to survive in mouse brain and to display a variety of neural markers. This study suggested that exfoliated deciduous teeth may be an unexpected unique resource for stem cell therapies including autologous stem cell transplantation and tissue engineering. Also, we have further demonstrated self-renewal capability, multi-potential differentiation, and have identified the specific niche occupied by human dental pulp stem cells in vivo to be perivascular surfaces, similar to BMSSCs.

The Skeletal Clinical Studies program, currently under the direction of Dr. Pamela Gehron Robey, has continued to focus on two aspects: the role of BMSSCs in skeletal disease, and the use of BMSSCs for tissue regeneration. Patient accrual is continuing in four clinical protocols (97-DK-0055, 98-D-0145, 98-D-0146, 00-D-0183) for the study and treatment of fibrous dysplasia of bone (FD) and the McCune-Albright Syndrome (MAS), which arise from a post-zygotic mutation in the GNAS1 gene (R201C and R201H). We have previously noted that there is a great deal of variability in the histological appearance of FD lesions taken from different patients, and hypothesized that some of these changes may be related to the metabolic status of the patient. Histology, histomorphometry, and quantitative back-scattered electron imaging analysis demonstrated a marked excess of unmineralized osteoid (osteomalacia) with a negative correlation between osteoid thickness in lesional bone and renal tubular phosphate reabsorption, but not for unaffected bone from the same patient. Increased bone resorption was variable in lesional bone, and clearly correlated with serum levels of PTH. Hyperparathyroidism-related histological changes were observed in FD bone, but not in the unaffected bone, of patients with elevated serum PTH. Our data indicate that osteomalacic and hyperparathyroid changes, which emanate from distinct metabolic derangements (which superimpose on the local effects of *GNAS1* mutations in bone), influence in turn the severity and type of skeletal morbidity in FD. FD frequently involves the anterior base of the cranium and results in encasement of the optic-nerve canals, and it has been assumed that such encasement leads to constriction and eventual blindness. We studied patients with FD of the lesser wing of the sphenoid bone and found that in 73% of the optic canals, there was complete encasement. In all but two of the patients, the results of neuro-ophthalmologic examination were normal. In the two patients with monocular visual impairment, the areas of the optic canals were similar on the normal and abnormal sides. Encasement of the optic canal by FD bone caused ~18 % narrowing of the canal, but that in itself does not result in visual loss. Therefore, prophylactic decompression of the optic nerve does not appear to be indicated on the basis of the presence of FD on diagnostic images alone, since it does not correlate with visual loss. Finally, FD is also frequently found in the maxilla and mandible, but its effects on dental tissues and implications for dental care have not been well delineated. We characterized dental features associated with maxillo-mandibular FD and reaction of affected bones to routine dental therapy. While patients with craniofacial FD present with a higher incidence of caries and dental malformations, they did not require special dental management and were able to undergo routine dental care. The group is also progressing towards another IND application to the FDA for the use of ex vivo expanded bone marrow stromal stem cells to be used in bone regeneration in the context of fibrous dysplastic lesions.

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# Gene Therapy and Therapeutics Branch

Bruce Baum
Indu S. Ambudkar
John Chiorini
R. James Turner

# GENE THERAPY AND THERAPEUTICS BRANCH
## 2003

The Gene Therapy and Therapeutics Branch (GTTB) is a model of translational research with a bench to clinic continuum. The GTTB has a primary tissue-specific focus, asking questions related to salivary gland biology, pathology and management, as well as a primary applications focus, gene transfer technology. Saliva is the principal protective agent for the mouth and upper GI tract and thus is of critical importance to health maintenance. Perturbations of salivary secretory mechanisms can lead to serious oral and general health problems. The GTTB is committed to the notion that significant advances in clinical care will come from our understanding of biological mechanisms and an inter-disciplinary approach to problem solving. This reporting period has seen our continued and substantial scientific progress.

The production of salivary fluid is due to neurotransmitter stimulation of transepithelial secretion of $Cl^-$ by acinar cells. A $Na^+$-$K^+$-$2Cl^-$ cotransporter, NKCC1, located in the acinar basolateral membrane drives much of this $Cl^-$ flux and this transporter is the rate-limiting step in salivary secretion. The Membrane Biology Section (MBS) has long concentrated on understanding the structure, function and regulation of this important transport protein. Over the past year, MBS studies addressed the transmembrane topology and biogenesis of NKCC1 using a new method that they recently developed. This method involves the construction of a fusion protein consisting of the green fluorescent protein followed by a portion of the membrane spanning region of NKCC1 and a C-terminal glycosylation tag. By assaying for glycosylation of this tag, MBS scientists can determine whether it is located in the lumen of the endoplasmic reticulum or the cytosol. By systematically varying the length of the NKCC1 membrane-spanning region included in the construct, NKCC1's biogenesis and topology can be traced as it integrates into the membrane. NKCC1 contains 12 hydrophobic regions (HRs) that previous MBS studies indicated were membrane-spanning segments. This year, several additional and important insights were established. For example, the highly conserved intracellular loop between HRs 2 and 3 of NKCC1 appears to be membrane inserted. Secondly, the sixth HR of NKCC1 requires the presence of the seventh and eighth HRs in order to integrate into the membrane, suggesting a specific interaction of those HRs during the intramembrane folding of NKCC1. Finally, glycosylation of endogenous sites in NKCC1 located between the seventh and eighth HRs does not occur until after all 12 HRs of the protein are integrated into the membrane, indicating that a maturation event is necessary before these sites are made available to the glycosylation machinery within the endoplasmic reticulum.

Capitalizing on this methodology, MBS scientists examined the structural properties of presenilin 1, the putative proteolytic component of gamma-secretase. Presenilin 1 is thought to be the protease that is responsible for the intramembrane cleavage of a number of substrates including the beta-amyloid precursor protein, the protein that is primarily responsible for the senile plaques characteristic of Alzheimer's disease, and it also is found in salivary glands. Presenilin 1 contains 10 HRs sufficiently long to be alpha-helical membrane spanning segments. Previous topology studies agree that the N-terminus of presenilin 1 is cytosolic and HR 1-6 span the membrane but HR 7 does not. However, the conformation of HRs 8-10 has remained controversial. MBS studies this year clearly indicate that HRs 8, 9 and 10 all are able to span the membrane. While these conclusions differ from previous proposals, the data actually reconcile rather than contradict most of the earlier observations.

The neurotransmitter stimulation of fluid ($Cl^-$) secretion from salivary glands described above is mediated via a biphasic elevation in cytosolic [$Ca^{2+}$]. There is an initial transient increase due to internal $Ca^{2+}$ release and a latter sustained increase due to $Ca^{2+}$ influx. GTTB's Secretory Physiology Section (SPS) is focused on understanding this $Ca^{2+}$ influx process, which appears to be mediated via store-operated $Ca^{2+}$ entry (SOCE), a pathway ubiquitously present in all non-excitable cells. However, the molecular mechanism for this biologically critical influx pathway has not yet been determined in any cell type. Recently, the transient receptor potential (*trp*) gene family of ion channel proteins has been proposed as molecular components of the store-operated $Ca^{2+}$ influx channel (SOCC). The physiological function(s) of the presently identified *trp* gene products has not yet been established. By expressing wild type and mutant forms of one important *trp* gene family member, TRPC1, in salivary gland cells, SPS scientists recently showed that TRPC1 is a molecular component of agonist (neurotransmitter)-stimulated SOCCs in salivary gland cells. For example, TRPC1 overexpression induced channel activity that was indistinguishable from the endogenous SOCC activity. Transfection with anti-sense TRPC1-cDNA or expression of a mutant TRPC1 (termed Δ567-793) decreased SOCC activity. Simultaneous substitutions of seven acidic amino acids in the proposed pore region of TRPC1 (548-611, D→N and E→Q) altered SOCC-mediated $Ca^{2+}$, but not $Na^+$, current.

Additionally, during this reporting period SPS scientists examined the role of caveolin-1 (Cav1) in the functioning of TRPC1. TRPC1 and Cav1 were co-localized in the plasma membrane region of HSG (human salivary) cells. Full-length Cav1 bound to both the N- and C-terminus of TRPC1. The region encompassing amino acids 271-349, which includes a Cav1 binding motif (at 322-349), was identified as the Cav1 binding domain in the TRPC1 N-terminus. Deletion of residues 271-349 or 322-349 prevented plasma membrane localization of TRPC1. These SPS data suggest that Cav1 has an important role in the assembly of SOCE channel(s). The mechanism(s) involved in assembly of TRP channels in the plasma membrane is also not known. This year SPS scientists showed that the *trp* gene family member TRPC3 can interact with the SNARE proteins VAMP2 and αSNAP via its N-terminus, and is co-localized with SNARE proteins in both epithelial and neuronal cells, suggesting these proteins facilitate TRPC3 localization and assembly.

Despite being the newest research group within the GTTB, the AAV Biology Unit (AAVBU) has made remarkable progress in its goal to understand the interactions of adeno-associated virus (AAV) with its host cell. Previous work by the AAVBU with AAV4 and AAV5 demonstrated distinct tropisms for these viruses compared to the more commonly utilized gene transfer vector AAV2. While heparin sulfate proteoglycans are important as cellular receptors for AAV2 binding and transduction, AAV4 and AAV5 interact with the cell surface by a distinct mechanism. AAVBU scientists identified sialic acid as an important cellular component in transduction with these serotypes and developed new tools to identify the genes involved in these events. During this year, using microarray technology, they linked AAV5 transduction with the expression of the platelet derived growth factor receptor alpha (PDGFRα) and beta (PDGFRβ). This highly novel discovery has helped define the biology of this serotype and likely will facilitate new applications for AAV5 as a gene transfer vector. AAVBU scientists also demonstrated a stable interaction between some of the non-structural proteins of AAV (the Rep proteins) and the important cellular signaling enzyme cyclic AMP dependent protein kinase, PKA, and its' novel homolog, PrKX. They showed that this interaction constitutes a novel form of viral interference and that one role of this interaction in the lifecycle of AAV is to control the replication of the adenoviral helper virus during a co-infection.

The Gene Transfer Section (GTS) studies clinically relevant applications of gene transfer technology to salivary glands. During this reporting period the GTS addressed three fundamental and practical questions necessary to move its gene transfer approaches into the clinic for phase I trials. The key questions addressed, and the answers obtained, were as follows. First, what is the best recombinant vector available to use for stable transgene expression in salivary glands? At present, the answer appears clearly recombinant AAV serotype 2 (rAAV2). In collaboration with the AAVBU, GTS scientists used a rAAV2 vector encoding human erythropoietin (hEpo) and showed the secretion of functional hEpo into the bloodstream for 54 weeks (last time point studied) in mice. Second, can transgene expression in salivary glands be regulated? Yes, as during this reporting period the GTS has shown that it is possible to regulate transgene expression using the rapamycin-based dimerizer drug system. Employing two adenoviral vectors to deliver the components of this transcription regulation system, GTS scientists showed that rapamycin is able to function as a control switch for transgene expression in rat salivary glands. Importantly, in the absence of rapamycin, no transgene expression is detected. Third, can results obtained in rodent model experiments be scaled to larger animals? Yes, and GTS scientists have recently shown that using a comparable multiplicity of infection (dose; adenoviral infectious units/μl infusate), similar levels of transgene expression in mouse and minipig salivary glands are obtained.

The GTS also extended studies initially reported last year examining whether bone marrow progenitor cells are capable of transdifferentiating into buccal mucosal and/or salivary epithelial cells in humans. Four to six years after male-to-female marrow cell transplantation, all five female recipients studied exhibited Y-chromosome positive buccal cells (0·8% to 12·7%) that were cytokeratin 13 positive. In > 9700 cells examined, only one XXXY positive cell (0·01%) and one XXY cell (0·01%) was detected, both of which may have arisen when a XY-cell fused with a XX cell. Thus, it appears that human male bone marrow cells migrate into the cheek and differentiate into epithelial elements, a phenomenon that does not depend on cell fusion.

Sjögren's Syndrome (SS) is an autoimmune disease, characterized as a widespread "epitheliitis", which results in dryness of the lining surfaces of the body, producing, most notably, dry mouth and dry eyes. GTTB's SS Clinic conducts clinical investigations and clinical trials, and also collaborates with laboratory investigators to elucidate pathogenic mechanisms operative in this disease. A major achievement during this FY has been the near completion of a secure, integrated, relational database incorporating research records since the inception of the SS Clinic in 1985. The entry of clinical data on ~1800 subjects, who have participated in studies of SS and salivary dysfunction, is almost finished. This database includes information on hundreds of variables from questionnaires completed by patients on history and symptoms; data forms completed by clinicians, such as physical findings and salivary flow rates; and information imported from the NIH Clinical Center's Medical Information System. The importation of this laboratory and pathology data relies on specific computer programs that automatically detect inconsistencies and duplicates. In addition, sophisticated algorithms have been developed within statistical computer programs to allow for the automated classification of cases to determine whether or not they meet accepted criteria of SS, and whether the cases are classified as primary or secondary SS. This data-base clearly will be a rich resource, allowing GTTB scientists and their colleagues rapid and convenient access to valuable research information. Previously, the SS Clinic hypothesized that systemically administered immunomodulatory treatments may favorably alter the course of SS. Accordingly, the SS Clinic conducted a placebo-controlled, randomized clinical trial (RCT) of a new biologic agent, etanercept, which recently was shown to be useful in treating rheumatoid arthritis patients. Etanercept is an inhibitor of (soluble receptor for) tumor necrosis factor α, a cytokine found in increased amounts in salivary and lacrimal glands of SS patients. This RCT was very recently completed, and interestingly the results show no evidence for efficacy of etanercept in treatment of the exocrine component of SS. Additionally, during this year, in collaboration with an international group of SS investigators, the National Eye Institute, the Office of Women's Health, the Sjogren's Syndrome Foundation, and industry, the SS Clinic led a major workshop to develop an international consensus on outcome measures for clinical trials in SS. From this endeavor, a framework was established for the validation of a core set of outcome measures, as well as for the evaluation of newer, potential outcome measures for which there are currently insufficient data.

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# Oral Infection and Immunity Branch

Sharon M. Wahl
John Cisar
Paul Kolenbrander
Abner Notkins
Nick Ryba
Reuben Siraganian
John Thompson

# ORAL INFECTION AND IMMUNITY BRANCH
# 2003

The Oral Infection and Immunity Branch (OIIB) plans, fosters and carries out research relating to the causes, diagnosis, treatment and prevention of infectious and inflammatory diseases. Efforts to understand the functional and molecular organization of infectious organisms, and research into the cellular, biochemical and molecular components of inflammatory, immune and sensory responses provide the basis for dissecting the interactions between pathogens, noxious agents and the host defense system. These multifaceted approaches define fundamental mechanisms of host defense, how these pathways become dysregulated in disease, and how to intervene for the benefit of the host.

The past year, considerable effort was invested in preparation, planning and presentation of our research portfolio to the NIDCR Board of Scientific Counselors in June 2003. The BSC applauded the Branch for its development of a strong research program and recognized the strengths of the Branch in our diversity, mentoring, revitalization of space, and our ongoing efforts to integrate the strengths of the individual investigators into each other's programs. During the past year, we also focused on the recruitment of new tenure-track investigators into the Branch. With the previous departure of two senior scientists from the Branch, opportunities for recruitment and new directions emerged, and two national searches for tenure-track investigators in mucosal immunity and in host-pathogen interactions were conducted. Based on the recommendations of the Search Committees and concurrence of the institute leadership, to date, one candidate was selected for the mucosal immunity tenure track investigator and the documentation to support the appointment is currently in progress. This is an exciting time for the Branch and we look forward to the opportunities for interaction and challenges by our new faculty.

Mentoring young investigators continues to be a major emphasis of the Branch. The cornerstone for our clinical training programs is a joint effort with Children's National Hospital Center, Department of Pediatric Infectious Diseases, in which their infectious disease fellows enroll in a three year program which includes clinical service and a research fellowship experience in the OIIB. In addition, through a collaborative program with the University of Maryland Department of Periodontics, a fellow in periodontics is pursuing graduate training with a research component in the OIIB. Similarly, an oral surgery fellow from the Department of Oral Surgery at the University of Maryland is also being mentored in his first research experience in our Branch. A collaborative effort between the Johns Hopkins Department of Molecular Microbiology and Department of Clinical Trials and the OIIB has resulted in successful completion of a clinical trial and more importantly, culminated in the awarding of a PhD in Clinical Trials for Dr. Sharon Gordon. An important outcome of this project was the identification of a new mode of management of aphthous ulcers in immunosuppressed individuals, which has become the foundation for a pending multicenter clinical trial. The Branch has defined a critical niche in the training of clinical investigators which will help to alleviate the insufficient levels of clinical research necessary to achieve the goals of prevention, diagnosis and management of oral, dental and craniofacial diseases. Promoting the integration of oral health research and basic immunology and microbiology research makes for an important mix of expertise to take advantage of the wealth of new information available through genomic and proteomic technology. Through these mentoring opportunities, senior investigators continue to generate diverse and exciting advances in our research portfolio, providing a well-spring for translational and clinical activities. During the course of our research endeavors, multiple novel reagents, antibodies, and transgenic animals are developed and members of the Branch serve as a resource for NIH and investigators worldwide for these reagents and intellectual input. Our senior investigators are continually sought out for their expertise, to write scholarly reviews, to participate in committees and study sections and to organize and chair conferences and symposia.

In another productive year, the Branch has made numerous advances and remarkable progress in many of its multiple unique and diverse research programs, as reflected in the publications in our annual bibliography. Among the scientific advances, including those featured in prestigious journals such as *Cell*, *Journal of Experimental Medicine*, *Journal of Clinical Investigation* and *Journal of Biological Chemistry* are those highlighted in this 2003 Annual Report. Collaborations within the Branch and worldwide embellish the opportunities that are essential to advancing the understanding of complex infectious diseases and position the Branch to address major pathophysiologic questions, create new hypotheses, and tackle them through *in vitro* and *in vivo* analyses and clinical investigations. Detailed research summaries are provided by each senior investigator describing their accomplishments, and are summarized in the following paragraphs.

Aberrant immune regulation and/or failed tolerance mechanisms result in the development of autoimmunity, and the Branch has had a long standing interest in exploring the underlying basis for failed tolerance and emergence of autoimmune diseases such as diabetes, arthritis and Sjogren's syndrome. In this regard, Branch scientists have contributed to the development of diagnostic tools to screen and predict those at risk, providing the opportunity for detection and prevention. Moreover, the self-antigens, which have been identified as autoantibody targets, are providing new clues as to the underlying genetic, cellular and biochemical basis of this disease. In related studies, investigators have identified multiple contributing factors to the establishment of chronic destructive arthritis in a pre-clinical animal model and importantly, developed therapeutic targeting strategies to ameliorate the inflammation and attendant tissue destruction. Recent evidence that local, rather than systemic, gene transfer of a TNFα receptor antagonist attenuates the disease process without dose limiting systemic side effects offers novel insight into therapeutic considerations. At a more fundamental level, investigators have zeroed in on a population of suppressor T cells ($CD4^+CD25^+$ T cells) that contribute to peripheral tolerance and the control of autoimmunity. Defining how these cells work, their source and how to regulate them is an emerging area of immunology about which there is much excitement due to the potential to regulate these cells in the control of autoimmunity, cancer and transplantation.

A significant component of the OIIB research portfolio includes an exploration of infectious microorganisms and the host response to these bacteria and viruses. In this regard, pathogens that colonize the oral cavity are being studied for their ability to contribute to disease, including caries, either as individual pathogens or as colonies or biofilms. From this research, a new paradigm in the metabolism of sucrose by specific microorganisms calls into question established dogma in this field. Branch scientists have elegantly defined the biochemical basis for bacteria-bacteria interactions and how these complex carbohydrate interactions promote both intra- and interspecies communication. In the Branch, investigators have defined the mechanisms of interaction among different bacterial species in the oral cavity and are exploring how these interactions function in the development of communities and disease manifestations. This represents a new concept in terms of our understanding that initial dental plaque is composed of mixed species communities, that these communities are formed by coaggregation-mediated events, and that these polymicrobial communities may be etiologic agents. Communication among the species of oral biofilm bacteria is being investigated by identifying soluble signaling molecules. In addition to the ability of microorganisms to form a community for self-preservation, microbes have other clever mechanisms of self defense. In this regard, *M. avium*, an intracellular opportunistic pathogen in immunocompromised HIV-infected individuals, cleverly co-opts macrophage signaling pathways to its own benefit enabling survival and replication. HIV remains a major killer with nearly 40 million infected individuals worldwide and as resistant strains continue to emerge, added emphasis must be given to host-derived targets to blunt this raging disease. To this end, experimental approaches have utilized cDNA microarray technology to identify virus-regulated macrophage genes that can be targeted to inhibit HIV infection. As an example, efforts have focused on an endogenous inhibitor of HIV, secretory leukocyte protease inhibitor (SLPI), which targets host cells, rather than viral products. The roles of many of the bacterial and viral genes are yet to be determined, but with the new technological approaches in proteomics, *in vivo* expression and regulation, and transcriptional profiling, new insights into the pathophysiology of infections at the level of the pathogen and the host will be forthcoming. Such insight will drive diagnostics, drug discovery, surveillance, control, and prevention strategies.

Whether in the oral cavity or other sites, the host response to infection or other antigens involves activation and signaling events in inflammatory cells. An elaborate unraveling of the complexities of signal transduction leading to release of inflammatory mediators has opened new opportunities for disrupting these activation sequelae, with the definition of which intracellular targets are essential and which are redundant. Intracellular signaling via cell surface receptors represents the backbone of cellular function and an important focus continues on identification of receptors and their signaling pathways, which control the senses of taste and smell. In a stunning series of papers, work in the Branch has changed and challenged the entire field of chemosensory perception. In the broader context, these studies also provide a means of augmenting our knowledge of host defense, including rejection or avoidance of toxic or noxious agents.

Collectively, our unique assemblage of investigators in the Oral Infection and Immunity Branch has been exceptionally productive, uncovering new knowledge that can change the landscape of modern science. Our multidimensional approach to complex problems offers new insight that can tangibly translate into medical/dental practice. As has been our hallmark for the past 50 years, the Oral Infection and Immunity Branch will maintain its commitment to excellence in research, in mentoring and in outreach.

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# Oral and Pharyngeal Cancer Branch

J. Silvio Gutkind
Thomas Bugge
Myung Hee Park
Adrian Senderowicz

# ORAL AND PHARYNGEAL CANCER BRANCH
## 2003

Cancer of the head and neck area is the sixth most common neoplastic disease in the developed world, representing a very serious public health problem based on annual morbidity and mortality rates. The molecular and etiological factors involved in the development of head and neck tumors, including oral cancers, are still not fully understood. Members of our Branch work on complementary basic, translational, and clinical aspects of cancer research, in an effort to understand the molecular basis for malignant transformation as well as tumor invasion and metastasis, and to use this knowledge for the development of molecular markers of disease progression and novel therapeutic approaches for oral malignancies.

During the current reporting period, we have continued to expand the breadth and depth of our program by making a concerted effort in areas of direct relevance to head and neck cancer research. This includes the continuing efforts of Dr. Myung Hee Park, Dr. Thomas Bugge, Dr. Adrian Senderowicz, and Dr. J. Silvio Gutkind's teams, whose research programs address the most basic molecular mechanisms participating in normal and aberrant cell proliferation, tissue remodeling, squamous carcinogenesis and tumor metastasis, as well as the development and evaluation of novel mechanism-based therapeutic approaches for head and neck cancer patients. These directions are expected to provide a better understanding of the molecular alterations that lead to oral malignancies, thus broadening the horizon on developing potential oral tumor markers and treatments modalities.

Our Branch has been highly productive during this year, and has made significant contributions to the field, providing new concepts and shedding new light on questions of fundamental importance for cancer biology. We have also continued developing a large number of novel reagents of value to biomedical research, such as new genes, expression vectors, cell lines, gene arrays, modified toxins, and engineered mouse strains, and provided them to hundreds of investigators in the U.S. and abroad. Another tradition of our Branch has been our high priority on mentoring students and the training of postdoctoral investigators to become independent leaders in the field; we have tried to continue and strengthen this commitment.

During the current reporting period, significant progress has been made in a number of research efforts at the OPCB. A variety of arbitrarily selected research advances are highlighted below. The progress report for each project provides a more comprehensive description of the major findings in our Branch.

The completion of the human genome project and the recent development of novel, highly sensitive high-throughput techniques have now afforded the unique opportunity to perform a comprehensive molecular characterization of normal, precancerous, and malignant cells. As part of these efforts, a joint program was established between NCI and NIDCR aimed to identify the nature of those genes that are expressed during HNSCC development. The use of bioinformatic tools to analyze the database of cDNA libraries derived from microdissected normal and HNSCC tissues contributed by our laboratory helped identify a large number of potential novel genes, and their differential expression in normal and tumor tissues was examined using cDNA microarrays. A systematic characterization of gene expression profiles in a large collection of HNSCC cell lines and normal and transformed human oral keratinocytes was also performed. Genes primarily involved in cell cycle regulation, oncogenesis, cell proliferation, differentiation, apoptosis and cell adhesion were found to be widely altered in all HNSCC lines studied. Up-regulated genes included known oncogenes, protein kinases, DNA binding proteins and cell cycle regulators, while those commonly down-regulated included differentiation markers, cell adhesion proteins, extracellular matrix proteins, structural proteins (keratins) and protease inhibitor proteins. By hierarchical clustering analysis, we found two distinctive subtypes of gene expression patterns among the cell lines, suggesting that these subgroups might have undergone different carcinogenesis pathways. In addition, using laser capture microdissection and recently developed linear amplification techniques, we are now examining gene expression patterns in normal and tumor tissues, focusing in SCC from the tongue, a major oral cancer site, and carcinomas of the nasopharynx. In parallel, efforts are currently underway to use serial analysis of gene expression (SAGE) to catalog HNSCC transcribed genes (HNSCC transcriptome). These efforts, together with other multi-institutional initiatives, are likely to contribute to the complete understanding of the molecular pathogenesis of HNSCCs, thus helping to identify new markers for the early detection of preneoplastic lesions and novel targets for pharmacological intervention.

Proteolytic modification of the extracellular matrix is essential for physiologic tissue remodeling, as well as for the progression of a number of chronic, degenerative diseases including cancer invasion, and metastasis. We have addressed the biochemistry, biology, and pathology of selected cell surface-associated serine proteases, in particular, their relation to the development, regeneration, and malignant transformation of keratinized epithelium. Studies included the role of the plasminogen activation (PA) system in cancer development. In this regard, attempts to identify the initiation routes and the sites of plasminogen (Plg) activation during tissue remodeling *in vivo* are hampered by the lack of suitable systems for detecting the activation state of the minute quantities of Plg activators that are present in living tissues. In collaboration with the MPS, NIAID, we have performed proof of concept studies to demonstrate that engineered urokinase Plg activator (uPA)-activated cytotoxins can serve to detect cell surface uPA activity *in vivo*. These studies have established that the uPA receptor (uPAR) is critical for generating cell surface uPA activity *in vivo*, that plasmin is critical *in vivo* for the efficient conversion of pro-uPA to active uPA, and that Plg activator inhibitor-1 actively restricts the generation of cell surface uPA activity *in vivo*. We are continuing the development of modified cytotoxins as imaging agents for cell surface protease activity. We have also identified two novel cell surface serine proteases that are involved in epidermal development, repair, and malignancy. These are members of a family of membrane anchored serine proteases, the type II transmembrane serine proteases (TTSPs). For example, we have studied in detail Matriptase/MT-SP1. We have found that this novel protease has pleiotropic functions in epidermal development and is required for epidermal barrier function, hair follicle development, and thymic homeostasis.

Receptors exhibiting an intrinsic tyrosine kinase (RTKs) activity or that are coupled to heterotrimeric G proteins (GPCRs) can effectively stimulate growth promoting pathways in a large variety of cell types and, if persistently activated, these receptors can also behave as dominant-acting oncoproteins. Indeed, it was recently found that the Kaposi's sarcoma virus (KSHV/HHV-8), the infectious cause of Kaposi's Sarcoma (KS) which is the most frequent cancer arising in HIV-infected patients and oral neoplasm in immunosuppressed patients, encodes numerous potential oncogenes, including a constitutively active GPCR (vGPCR). To begin addressing their contribution to KS we developed a novel transgenic animal model enabling endothelial cell-specific infection *in vivo* using avian viruses expressing candidate

KSHV oncogenes. Remarkably, retroviral transduction of v*GPCR,* was sufficient to induce angioproliferative tumors resembling KS. Additional data supported that vGPCR cooperates with latent KSHV genes to promote tumorigenesis, thus implicating vGPCR in both the initiation and promotion of KS. How this and other GPCRs promote tumorigenesis is under investigation. In this regard, the ability to stimulate GTPases of the Rho family appears to be a central component of the mitogenic and transforming pathway utilized by these receptors. Work in our laboratory and others led to the identification of a novel family of guanine-nucleotide exchange factors (GEFs) that provides a direct link between heterotrimeric G proteins of the $G_{12/13}$ family to Rho stimulation. The molecular mechanisms regulating the function of these GEFs and Rho GTPases in signaling by GPCRs and Plexins, which are receptors for the axon guiding molecules semaphorins, has been investigated. However, which genes are in turn regulated by Rho proteins, and how do they contribute to cell growth is not known. To address this issue, we investigated the global gene expression pattern induced by Ras and Rho GTPases by cDNA microarray analysis, which led to the identification of key genes that are up- or down- regulated with a pattern both unique and common to Ras and Rho proteins. Among them, the underlying mechanism by which RhoA stimulates the expression of the c-*jun* proto-oncogene has been studied in detail. These results will provide important information to begin unraveling the complexity of the molecular mechanisms underlying the transforming potential and cell cycle effects induced by GPCRs and these small GTPases.

We have expanded our drug evaluation effort at the NIDCR, whose goal is to develop novel therapies aimed at improving the quality of life and life expectancy of oral cancer patients. We have focused on novel molecules affecting the cell cycle and angiogenesis, and exploited characteristics of cancer cells to develop novel cytotoxic agents. Two drugs, UCN-01, a novel protein kinase C (PKC) inhibitor, and perifosine, a novel alkylphospholipid with antitumor properties, were found to exhibit potent antiproliferative effects in HNSCC cells. For example, UCN-01 caused a $G_1$/S arrest and apoptosis in all HNSCC lines tested, including radiation-resistant cells, and displayed a very significant antitumor response in *in vivo* xenograft studies using a HNSCC model. Based on these findings and the encouraging results from the first Phase I trial of UCN-01 in patients with refractory neoplasms, which was performed in collaboration with NCI, we can conclude that UCN-01 may represent a good candidate for its evaluation HNSCC patients in a Phase II clinical setting. Ongoing basic studies on the mechanism of action of UCN-01 and perifosine may further facilitate their biochemical assessment in tissues from patients during the course of future clinical trials. Parallel efforts have focused on flavopiridol, a novel cdk inhibitor with potent antiproliferative activity in *in vitro* and *in vivo* models of HNSCC. The basic mechanisms by which flavopiridol induce a pro-inflammatory syndrome, a dose limiting toxicity in humans, was investigated, leading to the identification of IL6 as the most likely candidate. We are currently accruing patients for the NIDCR/NCI/NIDCD Phase II trial of daily bolus flavopiridol in HNSCC patients. In addition, as the acquisition of uPA activity is a hallmark of human malignancy, we exploited this tumor-related activity to design novel anticancer approaches by engineering, in collaboration with MPS, NIAID, an anthrax toxin that is specifically activated by cell surface uPA. The engineered toxin was found to display limited toxicity to normal tissue but potent tumoricidal activity that was strictly dependent on tumor cell surface PA, showing that a simple change of protease activation specificity converts anthrax toxin from a highly lethal to a potent tumoricidal agent.

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# Pain and Neurosensory Mechanisms Branch

Raymond Dionne
Michael Iadarola
Mitchell Max

# PAIN AND NEUROSENSORY MECHANISMS BRANCH
# 2003

The mission of the Branch is to increase understanding of the mechanisms underlying pain and inflammation, improve the diagnosis and treatment of chronic orofacial pain, and to disseminate this information to the biomedical community with emphasis on the needs of the dental profession. The PNMB is now making the transition from a broad program of basic and clinical research to a more focused translational program targeting the molecular-genetic mechanisms that contribute to the development of pain chronicity in the orofacial region and analgesic interventions to prevent or reverse these mechanisms. Rational drug development has failed to produce a single breakthrough in efficacy, despite many successes in the laboratory, supportive of the need for alternative approaches to analgesic research to optimize opportunities for translating scientific advances into improved pain relief through the development of more effective and safer treatments. The PNMB remains the only NIH intramural research program focusing on the neurobiology of pain and analgesia. As such, we are uniquely poised to capitalize on findings and mechanisms emerging from the intramural neuroscience community (>200 investigators and fellows), the resources of the Clinical Center, our network of collaborations both on campus and nationally, and the technological advances inherent in the fields of genomics and proteomics.

### *Hypothesis Generating Basic Studies*

The research program of the NGEU addresses basic molecular and physiological processes of nociceptive transmission in the central nervous system and translational investigations of new pain treatments. The molecular research is performed in animal models with emphasis on the molecular and neuronal elements at the beginning of the pain pathway. This includes molecules in the primary afferent nerve ending that sense painful stimuli and neurons in the dorsal spinal cord. The translational research involves several strategies for pain control: specific cell deletion for pain control based on reagents that that will eliminate only the pain-sensing neurons from either the dorsal root ganglion or the spinal cord and completely interrupt further pain transmission to the brain. Another approach utilizes medicinal and combinatorial chemistry, combined with molecular cell biology of pain sensing molecules, to identify new small molecule analgesics. The goal is to use these compounds in clinical trials to treat pain and at the same time obtain a better understanding of pain mechanisms.

The NGEU's translational program for pain therapy uses basic science findings to create new treatments for chronic pain. While multiple target areas emerged from bench research, the current program focuses on the two closest to clinical application. The first is use of vanilloids to delete pain-sensing cells from the dorsal root ganglion. An initial paper (Szabo et al, 1999) demonstrated that epidural application of vanilloids deletes primary afferent pain cells and produces a long-term analgesia. This was subsequently extended to demonstrate that (a) intraganglionic injection, which places the agonist directly around the cell body and (b) intrathecal injection which places the agonist around the spinal cord and affects many ganglia at once, also deletes pain cells and produces analgesia. The loss of pain sensitivity is evident in a variety of behavioral assays especially those mediated by C-fiber neurons which express high levels of TRPV1. Studies were performed in normal dogs to obtain an initial dose response and then in dogs with cancer or arthritic pain. The animals showed a profound relief from the pain aspects of their disease. This canine clinical trial was a strong impetus to our human clinical trial. The routes of administration used in the animal studies can be used in humans and the direct ganglionic injections may be especially effective when administered under CT or MR imaging guidance. RTX may provide two new ways to treat cancer pain, trigeminal and post-herpetic neuralgia, and other types of inflammatory and neuropathic pain conditions. Pain-sensing nerve ending can be temporarily blocked by application to the skin or site of surgical incision. RTX is extremely effective at blocking acute pain and pain from inflammation when applied peripherally. This provides a new method for treating post-operative or traumatic injury induced pain. It was also demonstrated that the compound can be applied perineurally to obtain a very selective effect on hyperalgesia.

### *Translating from "Bench to Bedside"*

The ability to assess the therapeutic potential of a novel therapeutic strategy in humans is limited by the sensitivity of the human pain model to detect changes in biologically-relevant endpoints. Demonstration of shifts in the stimulus-response to experimental pain stimuli has been widely used to achieve sensitivity but may be irrelevant to the study of pain from tissue injury or nerve damage. The oral surgery model has proven useful for over 25 years for predicting the clinical utility for opioids and non-opioid drugs but is now largely used in multicenter studies to achieve rapid evaluation of investigational drugs for pharmaceutical studies. The PNMB has extended the oral surgery model to evaluate analgesic mechanisms, neuroendocrine responses to pain and intraoperative stress, and the relationship between local release of inflammatory mediators and pain reports of pain and analgesia. This approach serves as a paradigm for translation clinical trials to provide proof of concept for novel therapeutic strategies.

Arguably the only pharmacologic improvement for pain in the last decade has been the introduction of the selective COX-2 inhibitors as effective analgesics with a greater safety profile than dual COX-1/COX-2 inhibitors. Use of micro dialysis probes with measurement of perfusate for markers of COX-1 and COX-2 over the first few hours after oral surgery permits direct assessment of the *in vivo* selectivity of a COX-2 inhibitor in humans. While administration of ibuprofen suppresses products of both COX-1 and COX-2 at all time points in comparison to placebo, celecoxib only suppressed products of COX at time points associated with COX-2 expression. No effect was seen on thromboxane $B_2$ or prostaglandin $E_2$ over the first 60 minutes post-surgery. Parallel studies conducted in collaboration with the NGEU demonstrated expression of COX-2 at 60 and 120 minutes post-surgery with continued expression of COX-1 throughout the postoperative period, consistent with its role as a constitutive enzyme. These data not only support the selectivity of a COX-2 inhibitor in a human model of pain from tissue injury but also illustrate the sensitivity and versatility of the oral surgery model as a powerful tool for translational pain research.

### *Developing Novel Therapeutics for Chronic Pain*

Pharmacologic intervention in the management of chronic orofacial pain is usually considered adjunctive to physical treatment based on the assumption that these treatments (e.g. splints, physical therapy) will eventually correct the underlying pathophysiologic process. Palliative drug therapy of intractable pain is also considered as an indication for drug treatment when pain is poorly controlled following failed treatments, such as surgical interventions, or when no other treatment is available. Standard texts and expert opinion provide recommendations for the use of NSAIDs based on their efficacy for acute pain and related chronic pain conditions such as rheumatoid arthritis. The results of two previous studies, one by our group, could not separate the effects of placebo over time from the actions of ibuprofen or piroxicam. The introduction of the selective COX-2 inhibitors might produce greater efficacy than the traditional NSAIDs or permit chronic administration of maximal doses with less dose-limiting adverse effects. A recently completed study compared the effects of celecoxib, naproxen and placebo for patients with well-characterized TMD. Naproxen produced significant analgesia over the course of the evaluation in comparison to both placebo and celecoxib. Celecoxib did not demonstrate significant analgesia using a 100 mm visual analog scale but did result in a statistical trend ($P < 0.06$) using the criterion of 50% relief from starting pain in comparison to placebo. This outcome is supportive of the need to use responder analyses for evaluating the efficacy of drugs used for TMD as 50% pain relief is generally considered to be meaningful pain relief and, with the greater safety profile for coxibs in comparison the NSAIDs when given chronically, suggests that coxibs might have beneficial effects in some patients but with a lower risk of serious GI toxicity.

The approach to evaluating mechanistic hypotheses and novel therapeutics for chronic pain has traditionally involved administration of a maximally tolerated dose of a drug with activity predictive of an effect on a mechanism of interest, e.g. dextromethorphan for neuropathic pain. The failure of many pharmaceutical programs based on activity in rat neuropathic models to demonstrate activity in human trials has prompted reconsideration of the approach taken by PNMB investigators in the search for novel therapeutics for chronic pain. Studies done by the Clinical Trials Unit attempt to elucidate the neural mechanisms and principles of treatment of chronic pain syndromes, with particular attention to the drug treatment of pain caused by nerve injury. A new interest is the use of clinical genetic methods to identify new mechanisms and targets. This year a randomized drug trial in chronic sciatica, a condition with point prevalence of 8 million Americans, compared the new anticonvulsant medication topiramate to placebo. Topiramate inhibits postsynaptic currents triggered by AMPA/kainate receptors, and in addition may inhibit pain by enhancing chloride flux and sodium currents. In 29 patients who completed a crossover study, topiramate reduced pain by 20% compared with placebo, showing statistical significance on most pain outcome measures, but two-thirds of the patients with reduced pain chose to forgo long-term treatment because effective doses also caused frequent and severe sedation and changes in taste and appetite Ongoing trials in the same population include a placebo-controlled 2x2 factorial study of morphine and nortriptyline and a study of the effects of chronic treatment with static magnetic fields of various orientations worn on the back over the diseased nerve root.

The wide inter-individual response to nerve injury and analgesic drugs invites speculation on a genetic component to neuropathic pain. This is leading to a clinical genetics in a large cohort of patients with painful lumbar disc herniation and nerve root compression. The recently approved clinical protocol will collect DNA from up to 500 patients and examine candidate genes in the Laboratory of Neurogenetics, NIAAA. If successful, this study may provide a basis for larger genetic association studies in chronic pain that might increase understanding of the inter-individual variation in the development of chronic pain following nerve injury.

Working with the Johns Hopkins University Pain Research Group, the Clinical Trials Unit completed a collaborative clinical trial in 70 patients with post-herpetic neuralgia by directing the experimental design and the Johns Hopkins clinicians treated the patients. The study was the first to directly compare the most commonly used drug treatments. Novel findings were that morphine treatment was significantly superior to tricyclic antidepressant treatment at similar side effect levels, and that patients with deafferentation on quantitative sensory testing were the most likely to respond to either treatment. Research collaborations at NIH are also being developed with the Pain and Palliative Care group and with the intramural program in complementary and alternative medicine.

***Future Directions for Translational Pain Research***

The training, experience, and research interests of the three PNMB senior investigators are complimentary and provide an experienced nucleus for collaborative efforts, as well as semi-autonomous research within their own research units. We will continue to address the problems of chronic orofacial pain with several approaches based on the expertise of current staff and through eventual recruitment of collaborators, trainees and staff with complimentary expertise needed to provide national scientific leadership in the field of chronic orofacial pain. A chronic orofacial pain clinic has been scheduled to coincide with the Pain and Palliative Care Service outpatient clinic to evaluate patients for ongoing and planned studies of temporomandibular disorders. Continued progress in the development of investigational treatments for intractable pain (RTX, the substance P-Pseudomonas exotoxin fusion protein, gene transfer, and unforeseen novel strategies) will form the basis for small pilot studies in carefully selected patients to test the safety and potential effectiveness as alternatives to unrelieved pain or the risk of iatrogenic injury through surgical interventions. A significant part of the problem of inadequate treatment for chronic orofacial pain and the persistence of non-validated treatment modalities is the continued reliance on training in pain diagnosis and management by the dental school faculty with little or no advanced training in pain treatment or research. The current clinical research fellowship developed under the leadership of the Scientific Director and the Office of Education now has six fellows, with three of the current fellows engaged in pain research. The standardized didactic track for the trainees, requirements for developing their own clinical protocols, and the clinical and pain research environment is conducive to most of these fellows being placed into dental schools to foster a tradition of pain management and pain research that supercedes current practices. This long-term approach needs to be supplemented by continuing efforts at re-educating dental practitioners relying on inadequate training and research expertise to differentiate between popularized but unproven treatments for pain and validated, safe treatments. PNMB staff will continue to use traditional approaches to continuing education (talks, review articles, chapters, texts, etc) but are in the process of re-designing the Branch website to provide therapeutic updates, the latest results of our research that is relevant to treatment of orofacial pain, and as a possible link to web-based clinical trials at collaborative study sites. In addition, several chapters in Dr. Max's web-based text are devoted to diagnosis and treatment of chronic orofacial pain.

## PAIN AND NEUROSENSORY MECHANISMS BRANCH
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# Functional Genomics Unit

Ashok Kulkarni

# FUNCTIONAL GENOMICS UNIT
## 2003

The main research focus of Functional Genomics Unit (FGU) is to delineate molecular pathways involved in the disorders affecting craniofacial and dental systems as a result of genetic abnormalities and environmental factors. The Unit's major research approach is centered on functional analysis of the candidate genes. Since its inception in 1996, the key research work of the Unit has been concentrated on the following studies:

We continue to characterize molecular roles of cyclin dependent kinase-5 (Cdk5) in neuronal phosphorylation, neuronal migration and survival, and dopaminergic or glutamatergic signal transduction to gain insights into its involvement in brain development, psychomotor functions, learning and memory, and neurodegeneratgion. Our earlier studies revealed that Cdk5 null mice that exhibit perinatal mortality associated with abnormal stratification of the cerebral cortex and hippocampus along with cerebellar defoliation, ectopic facial nerve nucleus, abnormal accumulation of neurofilaments in the neuronal cell bodies and ballooned motor neurons. Further studies confirmed a typical inverted cortex in these mice with a special "cell autonomous" role of Cdk5 in the nervous system. We have also generated many kinds of Cdk5 "conditional" knockouts that exhibit abnormal gait, postures, reflexes and seizures. Our studies also indicated synergistic actions of Cdk5, reelin and mDAB in brain development, particularly in positioning of facial branchiomotor and inferior olive neurons, involvement of Cdk5 in glutamatergic signal transduction and neuronal apoptosis. In the recent set of studies, Cdk5 has been implicated in abnormal tau phosphorylation and in molecular mechanisms underlying cocaine addiction.

In the second project, we continue to work on Fabry disorder because of its unique nature as a painful and fatal metabolic disorder and the challenges it presents in developing much needed therapeutic approaches. Earlier, we generated null mice, which exhibit lipid inclusions in the target organs typically seen in Fabry patients. Subsequent studies revealed that the aging of these mice accentuates and bone marrow transplantation ameliorates the phenotpye of these mice indicating a potential for BMT as a therapy for some of the Fabry patients. Additionally, we identified greater prevalence of dental and oral defects in Fabry patients and mutant mice. Our recent studies indicate abnormal salivary gland function in the Fabry mice. We have now extended the present studies to other lipid disorders to study role of lipids in salivary glands and tooth biology.

In the third project, we have made swift advances in characterizing molecular roles of candidate genes in tooth development and disease. Amelogenins, mainly expressed by ameloblasts and secreted to form a major component of the enamel matrix, play an important role in enamel formation, and defects in which are implicated in amelogenesis imperfecta. Amelogenin null mice, recently generated in our laboratory, mimic amelogenesis imperfecta disorder. Presence of amelogenins in cementum, as shown by immunostaining using anti-amelogenin antibodies, had suggested a potential role for these proteins in cementum. The objective of our current study was to analyze the defects in the cementum in the amelogenin null mice in order to delineate the function of amelogenin during cementogenesis. Teeth of amelogenin null mice and their littermate controls at different ages were analyzed using light microscopy, electron microscopy and micro-radiography. EM analysis of the null teeth revealed cobbled roughness on their root surface whereas wild-type mice have a relatively smooth surface. Light microscopic analysis of tooth sections of the null mice displayed significant increase in the number of cementicles (~6 fold increase in the null mice). A number of lacunaes were noticed in the cementum of the null mice indicating resorption of cementum and dentin. Periodental-ligament fibers were frequently seen deeply embedded into these lacunaes. Interestingly, amelogenin splice variant was detected in periodontal ligament cells of the wild type mice and absence of which was associated with upregulation of RANKL, a key molecule. These findings suggest that amelogenin plays an important role not only in enamel formation but may also participate in cementogenesis. We continue to delineate in vivo roles of dentin sialophosphoprotein gene (dspp) in dentinogenesis. We have developed a transgenic animal model with a reporter gene (ß-galactosidase) under the control of 5.7 kb 5' flanking dspp and the analysis of these mice validates this promoter for tooth specific expression of the candidate genes. We have now generated dspp null mice that display a novel phenotype with numerous dentin defects associated with abnormal expression of ECM proteins and vitamin D receptors.

We also continue to study role of transforming growth factor-ß (TGF-ß) in inflammation, arthritis and tooth mineralization. Our ongoing studies indicate important nature of the autocrine role of TGF-ß isoforms and their involvement in tooth mineralization. We have also recently identified expression of numerous members of crystallin family and down regulation of their expression by TGF-ß1 in teeth. Our ongoing studies are focused on delineating role of TGF-ß signaling pathway and the downstream target genes involved in tooth development and disease.

Besides publishing extensively, our Unit distributes its research materials by licensing, donating to repositories, and providing numerous gifts to research colleagues. FGU has filed numerous invention reports and completed many Material Transfer Agreements with extramural researchers to provide mouse models and other reagents. FGU members continue to be invited as featured speakers at national and international meetings, as members of expert advisory panels and grant reviews, and they are also actively engaged in collaborations with members of the extramural research community. FGU members are also active in mentoring summer interns from local high schools.

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# Immunopathology Section

Larry Wahl

# IMMUNOPATHOLOGY SECTION
## 2003

Research in the Immunopathology Section focuses on the biological mediators and signal transduction pathways involved in the modulation of human monocyte functions that may contribute to the immunopathology associated with various inflammatory lesions. Connective tissue destruction is associated with many diseases, such as periodontal disease, atherosclerosis and rheumatoid arthritis, in which the monocyte/macrophage is a prominent cell. Since matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) are believed to play a major role in the destruction and remodeling of connective tissue, a major emphasis has been placed on how these enzymes and inhibitors are regulated. Research is also conducted on the role of MMPs and TIMPs in the regulation of cellular functions that are not directly related to connective tissue metabolism.

The degree of pathology associated with inflammatory lesions is most likely controlled in large part by cytokines and their interaction in the regulation of MMPs. Our previous work has demonstrated that either TNF-alpha or GM-CSF enhanced MMP-9 production by monocytes whereas both cytokines are required for the induction of MMP-1. We have recently focused on interferon (IFN)-gamma and its interactions with TNF-alpha and GM-CSF in the regulation of MMP-1 and -9 production by monocytes. While IFN-gamma alone does not induce monocyte MMP production, in the presence of GM-CSF it stimulates TNF-alpha that results in the synthesis of MMP-1. In contrast, IFN-gamma inhibits the induction of MMP-9 by TNF-alpha. The mechanism of this inhibition involves IFN-gamma mediated activation of caspase 8 that in turn activates p38 mitogen activated protein kinase (MAPK) that phosphorylates STAT1. Activated STAT1 induces IRF-1 that binds to IFN-gamma stimulated responsive elements, and as shown be other investigators, that overlap NF-kB binding sites in the MMP-9 promoter thus inhibiting MMP-9. Therefore, the NF-kB induced by TNF-alpha is blocked by IRF-1 from binding to the MMP-9 promoter. Thus, IFN-gamma in the presence of GM-CSF and/or TNF-alpha differentially regulates monocyte MMPs through induction of TNF-alpha and a novel mechanism involving caspase 8.

As described for IFN-gamma, multiple signal transduction pathway components can influence the regulation of MMP production. We have initiated a series of studies to determine if the PI-3 kinase pathway through its interaction with protein kinase B (Akt) is involved in the regulation of MMP-9 by activated monocytes since Akt can activate NF-kB. Inhibitors of PI-3 kinase suppressed the activation of Akt and NF-kB as well as the induction of MMP-9, but not MMP-1, by LPS or TNF-alpha and GM-CSF. Moreover, inhibition of Akt also inhibited the activation of NF-kB and the production of MMP-9. These findings demonstrate that the PI-3 kinase/Akt/NF-kB pathway can selectively regulate MMP-9 production by monocytes.

Monocyte chemoattractant protein-1 (MCP-1) recruits monocytes into inflammatory sites, such as atherosclerotic plaques and periodontal lesions, where monocyte derived MMPs may mediate the pathology associated with these lesions. The level of this protein in the blood is associated with a single nucleotide polymorphism in the promoter region of the MCP-1 gene (-2518). The G polymorphism at position -2518 causes a higher expression of MCP-1 protein compared to monocytes from A/A homozygous individuals. In studying the potential role of MCP-1 in the regulation of monocyte functions that contribute to disease we initially focused on atherosclerosis, since MCP-1 has been associated with symptomatic coronary heart disease (CHD). However, it is unknown whether the G allele is associated with earlier stages of coronary atherosclerosis in asymptomatic individuals and whether the adverse effect of the allele may be dependent on the presence of CHD risk factors. In a collaborative study with The Johns Hopkins University School of Medicine to determine whether MCP-1 polymorphism is associated with occult disease, we genotyped 676 healthy siblings (SIBS) of people with premature coronary heart disease and tested for the presence of occult ischemia with exercise treadmill tests (ETT) and exercise single photon emission computed tomography (SPECT). Each individual was assigned a CHD risk score with the risks including: male > 45 or female > 55; hypertension; current smoking; diabetes mellitus; body mass index (BMI) >= 30 $kg/m^2$; triglycerides ∃ 150 mg/dL; LDL-C ∃ 130 mg/dL; and HDL-C < 40mg/dL. Analysis of SIBS with 0-2 CHD risk factors slowed that the G polymorphism had no effect on occult ischemia, however for those SIBS with risk scores ∃ 3, the G allele conferred a higher prevalence of occult ischemia (27% vs. 17%, p=0.02). The study shows for the first time the role of the MCP-1 A (-2518) G polymorphism in coronary atherosclerosis in an asymptomatic population and demonstrates a strong interaction between this gene and the presence of CHD risk factors. A possible mechanism may be the activation of this gene by both systemic activators such as C-reactive protein (CRP) and local stimulators that are directly associated with the risk factors.

We are currently studying the effect of CRP and other risk factors on the production of MCP-1 by monocytes and the relationship of these interactions to the induction of MMPs. Our data demonstrate that stimulation of monocytes with LPS or TNF-alpha and GM-CSF results in the production of MCP-1 that can be increased further by CRP. Moreover, MCP-1 or CRP can also increase MMP production by activated monocytes. Additionally, when activated monocytes are exposed to both CRP and ox-LDL there is an additive increase in MMP production. These results show that activation of monocytes results in the production of MCP-1 that can attract additional monocytes to the site of inflammation as well as enhance MMP production. Thus, when other risk factors are present, such as CRP and/or ox-LDL, there is a further increase in MMP production by activated monocytes. These findings provide further information as to the biomarkers and their interaction in the regulation of monocyte MMP production. It is anticipated that these biomarkers will be relevant in the regulation of most chronic inflammatory lesions.

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# Matrix Metalloproteinase Unit

Henning Birkedal-Hansen

# MATRIX METALLOPROTEINASE UNIT
## 2003

The Matrix Metalloproteinase (MMP) Unit seeks to understand the role of MMPs and their inhibitors in physiologic and pathologic processes. Recently, we have focused our studies on processes which are dependent on remodeling or removal of the extracellular matrix.

### Intramembranous bone formation and skeletal growth are absolutely dependent on MT1-MMP dependent degradation of transient cartilages

Previous studies have causally linked the phenotypic defects including growth retardation, skeletal deformities, craniofacial defects, skin fibrosis, dwarfing and arthritis-like joint abnormalities of the MT1-MMP deficient mouse to inadequate degradation and remodeling of selected type I and type II collagen matrices. Studies conducted over the past year have yielded further insights into the bone phenotype of the MT1-MMP deficient mouse. Our observations indicate that intramembraneous bone formation takes place on a scaffold of cartilage either in the neurocranium, Meckel's cartilage (mandible, condyle, inner ear,) or part of the clavicle. This cartilage primordium never calcifies and is never resorbed by chondroclasts but is instead dissolved by an only recently recognized process that is absolutely dependent on MT1-MMP. This process couples dissolution of extracellular matrix to apoptotic cell demise and facilitates removal of cartilage in a distinctly different manner than that known in endochondral ossification. In MT1-MMP deficient mice the cartilage rudiment remains throughout life but selectively looses its proteoglycan content while the collagenous frame remains. A subset of cells in these cartilage rudiments maintain cell divisions and display markers of mature bone cells. These observations suggest that some chondrocytes may be programmed to osteogenesis upon their release from cartilage matrix under normal circumstances. Identical matrix dissolution in the cartilagineous precursor of the groove of Ranvier which surrounds the growth plate as a mantle, and is responsible for the longitudinal growth of cortical bone, is also observed. This provides a plausible explanation for why the animals are severely growth inhibited. While growth plates are seemingly normal at least for the first 40 days, the failure to dissolve cartilage in the groove of Ranvier prevents timely longitudinal growth of the long bones. Moreover, we have discovered that the remodeling of soft tissue attachments (tendons, ligaments, joint capsules) is also blocked in MT1-MMP deficient mice, leading to gross pathology in these areas during skeletal growth.

In normal bone, osteocytes communicate through an extensive and elaborate set of cellular processes lodged in narrow cannaliculi. In collaboration with Dr. Robin Poole in Montreal, we have shown that the cell processes adjacent to the unmineralized cannalicular wall expresses both MT1-MMP and MMP-13 (collagenase 3). Analysis of animals deficient in these two enzymes show virtually complete absence of osteocyte processes in MT1-MMP deficient mice, but not in MMP-13 deficient mice, suggestion that formation and maintenance of the osteocyte process network structure is absolutely dependent on MT1-MMP.

### MT1-MMP deficiency leads to stunted root development and blocks molar eruption.

In collaboration with Dr. Wouter Beertsen in Amsterdam we have observed that cells of the periodontium phagocytize large quantities of collagen fibrils, but apparently are not able to metabolize it further in the absence of MT1-MMP. This failure to remodel type I collagen of the

Sharpey's fibers results in the molar root development is greatly stunted and the teeth fail to erupt.

### A second (and MT1-MMP-independent) pathway for degradation of collagen fibrils

Previous work has demonstrated the pivotal role of matrix metalloproteinase MT1-MMP [MMP-14] in MMP activation and fibrillar collagen turnover in fibroblasts. Here we demonstrate an alternative cell surface-associated pathway for MMP activation and collagen dissolution that utilizes the plasminogen activation (PA) cascade and is independent of MMP-14. MT1-MMP deficiency did not affect collagen dissolution. Keratinocytes deficient in either uPA or tPA also retained the ability to breakdown collagen. Interestingly, however, collagen dissolution was completely eliminated in keratinocytes with combined deficiency in both uPA and tPA. Combined PA deficiency also completely blocked the activation of MMP-13 by keratinocytes. In wild type keratinocytes the activation MMP-13 could be prevented by exogenously added PAI-1 and aprotinin, but not by TIMPs, suggesting that plasmin activates MMP-13 directly. We propose that the PA cascade promotes keratinocyte-mediated collagen breakdown via the direct activation of MMP-13 and possibly other fibrillar collagenases.

### MMP-20 (enamelysin) deficiency leads to severe enamel abnormalities in the mouse.

Previous studies have shown that enamelysin (MMP-20) is expressed only by odontoblasts and ameloblasts, and only during certain stages of tooth development. In collaboration with Dr. John Bartlett at Forsyth Research Institute, Boston we have uncovered a series of structural abnormalities and deficiencies in enamel formation, including spontaneous fracture of enamel from dentin resulting in widespread loss of enamel, particularly on the molar crowns.

## MATRIX METALLOPROTEINASE UNIT
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# Molecular Structural Biology Unit

Dennis Torchia

# MOLECULAR STRUCTURAL BIOLOGY UNIT
# 2003

The principal research goal of the Molecular Structural Biology Unit (MSBU) is to elucidate the structure and dynamics of proteins and associated molecules at the molecular level in order to provide a basis for understanding function. The main research tool used in this work is high resolution, multidimensional, nuclear magnetic resonance (NMR) spectroscopy. Recent activity has focused upon HIV-1 protease, as either the free enzyme or bound to a high affinity protease inhibitor. Progress that has been made during the past year, relating the structure and dynamics of these molecules with their functions is discussed below.

HIV-1 protease

The active HIV-1 protease is a homodimer made up of monomers containing 99 amino acid residues. All drugs directed against the protease have targeted the substrate-binding site of the dimer. Although at least six drugs have been developed that binding tightly to the active site, one unfortunate consequence of targeting a single drug binding site on the protease has been the emergence of viral strains which carry multi-drug resistant mutations on their protease constructs. For this reason, there is considerable interest in identifying other protease sites that are suitable drug targets. Although the protease monomer is completely inactive, we have demonstrated that it is folded and therefore contains numerous potential alternative drug-binding sites, making it an attractive anti-HIV target. In order to overcome problems with monomer aggregation, we have designed proteins in which the N- and C-terminal regions are linked by intra-monomer disulfide bonds. In particular, cysteine residues were introduced at positions 2 and at either 97 or 98 of the protease amino acid sequence and using NMR have shown that the Q2C/L97C monomer construct exhibits a fold similar to that observed for the monomer subunit in the active dimer. It is anticipated that monomeric proteases of this kind will aid in the discovery of novel inhibitors that bind to the monomer at the dimerization interface, rather than at the active site. Such inhibitors could circumvent the problem of multidrug-resistance invariably observed with current HIV-1 protease drugs all of which bind at the active site.

We have extended these studies of the protease monomer by using NMR to determine the first solution structure of a protease monomer, the HIV-1 protease spanning the region Phe1-Ala95 ($PR_{1-95}$). Except for the terminal regions (residues 1-10 and 91-95) that are disordered, the tertiary fold of the remainder of the protease monomer is essentially identical to that of the individual subunit of the active protease dimer. In the monomer, the side chains of buried residues stabilizing the active site interface in the dimer, such as Asp25, Asp29, and Arg87, are now exposed to solvent. The flap dynamics in the monomer are similar to that of the free protease dimer. We also show that the protease domain of an optimized precursor flanked by 56 amino acids of the N-terminal transframe region is predominantly monomeric exhibiting a tertiary fold that is quite similar to the $PR_{1-95}$ structure. This explains the very low catalytic activity observed for the protease prior to its maturation at its N-terminus as compared to the mature protease, which is an active stable dimer under identical conditions. An addition of even 2 amino acids to the N-terminus of the mature protease significantly increases its dissociation into monomer. Knowledge of the protease monomer structure and critical features of its dimerization may aid in the screening and design of compounds that target the protease prior to its maturation from the Gag-Pol precursor.

We have advanced NMR methodology for studying slow protein motions (on the millesecond to microsecond timescale) in solution by developing pulses sequences that measure the relaxation dispersion of amide proton and backbone carbonyl spins in proteins. These measurements together with measurements of NMR lineshapes, carried out at temperatures ranging from 2 to 28 °C, and measurements of hydrogen-deuterium exchange are being analyzed with the goal of developing a detailed model of the conformational fluctuations that occur at the dimer interface of the protease, both when it is free and when it is bound to a potent inhibitor.

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